In re Patent Application of: Takashi Tanaka et al.

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For: PRODUCTION OF PHYSIOLOGICALLY ACTIVE PROTEINS USING GENE RECOMBINANT SILKWORMS

TRANSLATOR'S DECLARATION

Honorable Commissioner of Patents & Trademarks
Washington, D.C. 20231

Sir:

I, Tsumoru Fukumoto , residing at c/o SEIWA PATENT & LAW, Toranomon 37 Mori Bldg., 3-5-1, Toranomon Minato-ku, Tokyo 105-8423, Japan declare the following:

(1) That I know well both the Japanese and English languages;

(2) That I translated Japanese Patent Application No. 2002-060374 , filed March 6, 2002 , from the Japanese language to the English language;

(3) That the attached English translation is a true and correct translation of the aforesaid Japanese Patent Application No. 2002-060374 to the best of my knowledge and belief; and

(4) That all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such false statements may jeopardize the validity of the application or any patent issuing thereon.

March 23, 2007
Date

Tsumoru Fukumoto
Translator Tsumoru Fukumoto

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[TITLE OF THE INVENTION] Cytokine Gene-Recombinant Silkworm and Method for Production of Its Protein

[NUMBER OF CLAIMS] 20

[INVENTOR]
[Address or Residence] c/o Nagoya Plant, Toray Industries, Inc. 9-1, Oe-cho, Minato-ku, Nagoya-shi, Aichi Pref.
[Name] Takashi Tanaka

[INVENTOR]
[Address or Residence] c/o Nagoya Plant, Toray Industries, Inc. 9-1, Oe-cho, Minato-ku, Nagoya-shi, Aichi Pref.
[Name] Shingo Hiramatsu

[INVENTOR]
[Address or Residence] c/o Nagoya Plant, Toray Industries, Inc. 9-1, Oe-cho, Minato-ku, Nagoya-shi, Aichi Pref.
[Name] Katsushige Yamada

[INVENTOR]
[Address or Residence] c/o National Institute of Agrobiological Sciences, 1-2, kannondai 2-chome, Tsukuba-shi, Ibaraki
[Name] Toshiki Tamura

[APPLICANT]
[Identification Number] 000003159
[Name of Applicant] TORAY INDUSTRIES, INC.

[APPLICANT]
[Identification Number] 501266419
[Name of Applicant] National Institute of Agrobiological Sciences

[PATENT ATTORNEY]
[Identification Number] 100077517
[Patent Attorney]
[Name of Patent Attorney] Takashi Ishida
[Phone Number] 03-5470-1900

[APPOINTED PATENT ATTORNEY]
[Identification Number] 100087871
[Patent Attorney]
[Name of Patent Attorney] Tsumoru Fukumoto

[APPOINTED PATENT ATTORNEY]
[Identification Number] 100082898
[Patent Attorney]
[Name of Patent Attorney] Masaya Nishiyama

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[NAME OF DOCUMENT] SPECIFICATION

[TITLE OF THE INVENTION] Cytokine Gene-Recombinant Silkworm and Method for Production of Its Protein

[SCOPE OF CLAIM FOR PATENT]

[CLAIM 1]

A method for production of a recombinant cytokine characterized by creating a gene-recombinant silkworm incorporating a cytokine gene in a chromosome, producing the recombinant cytokine protein in the silk glands or cocoon filaments of the obtained gene-recombinant silkworm, and then recovering the cytokine from the silk glands or cocoon filaments.

[CLAIM 2]

A method for production of a recombinant cytokine according to claim 1, characterized in that the cytokine gene linked downstream from a silk gland-specific expression promoter is incorporated into the chromosome.

[CLAIM 3]

A method for production of a recombinant cytokine according to claim 2, characterized in that the silk gland-specific expression promoter is the sericin gene promoter.

[CLAIM 4]

A method for production of a recombinant cytokine according to claim 2, characterized in that the silk gland-specific expression promoter is the fibroin H chain gene promoter.

[CLAIM 5]

A method for production of a recombinant cytokine according to any one of claims 1 to 4, characterized in that the cytokine gene is incorporated into the silkworm chromosome utilizing transposon-derived DNA.

[CLAIM 6]

A method for production of a recombinant cytokine according to claim 5, characterized in that the cytokine gene is positioned between two pairs of transposon-derived inverted

terminal repeats.

[CLAIM 7]

A method for production of a recombinant cytokine according to claim 5 or 6, characterized in that the transposon-derived DNA originates from an insect.

[CLAIM 8]

A method for production of a recombinant cytokine according to claim 7, characterized in that the transposon is piggyBac transposon derived from a *Lepidoptera* insect.

[CLAIM 9]

A method for production of a recombinant cytokine according to any one of claims 1 to 8, characterized in that the cytokine gene is an interferon gene.

[CLAIM 10]

A method for production of a recombinant cytokine according to claim according to claim 9, characterized in that the interferon gene is feline interferon-ω.

[CLAIM 11]

A method for production of a recombinant cytokine according to any one of claims 1 to 3, characterized in that the cytokine is extracted from cocoon filaments using a water-soluble solvent.

[CLAIM 12]

A gene recombinant silkworm having a cytokine gene transferred into a chromosome and having the nature of producing the cytokine in the silk glands or cocoon filaments.

[CLAIM 13]

A gene recombinant silkworm according to claim 12, characterized in that the cytokine gene transferred into the chromosome is an interferon gene.

[CLAIM 14]

A gene recombinant silkworm according to claim 13, characterized in that the interferon gene transferred into the chromosome is feline interferon-ω.

[CLAIM 15]

A vector for exogenous gene transfer into a silkworm chromosome, characterized by having a cytokine gene linked downstream from a silk gland-specific expression promoter.

[CLAIM 16]

A vector for exogenous gene transfer into a silkworm chromosome according to claim 15, characterized in that the promoter is the sericin gene promoter.

[CLAIM 17]

A vector for exogenous gene transfer into a silkworm chromosome according to claim 15, characterized in that the promoter is the fibroin H chain gene promoter.

[CLAIM 18]

A vector for exogenous gene transfer into a silkworm chromosome according to any one of claims 15 to 17, characterized in that the cytokine gene is positioned between two pairs of transposon-derived inverted terminal repeats.

[CLAIM 19]

A vector for exogenous gene transfer into a silkworm chromosome according to any one of claims 15 to 18, characterized in that the cytokine gene is an interferon gene.

[CLAIM 20]

A vector for exogenous gene transfer into a silkworm chromosome according to claim 19, characterized in that the interferon gene is feline interferon-ω.

[DETAILED DESCRIPTION OF THE INVENTION]

[0001]

[Technical Field of the Invention]

The present invention relates to a method for production of recombinant cytokines utilizing silkworm having the cytokine genes incorporated into its chromosomes. The invention further relates to gene-recombinant silkworms having the nature of producing the recombinant cytokines in the silk glands or cocoon filaments, and to vectors for exogenous gene transfer into silkworm chromosomes in order to produce the recombinant

cytokines.

[0002]

[Prior Art]

Recent years have seen active research in production of recombinant proteins utilizing gene recombinant technology, and the obtained recombinant proteins have been applied in a wide variety of fields including medicine, diagnostic reagents, foods, chemical goods and the like. In most cases, gene recombinant proteins are produced by conventional methods which involve transferring a gene coding for the amino acid sequence into bacteria, yeast or mammalian cells such as COS cells, growing the cells in medium and then recovering the synthesized proteins. However, post-translational modification has been a problem in bacterial or yeast systems, and sometimes an adequately functional protein cannot be synthesized. Mammalian cells generally synthesize proteins in functional form, but growth is usually difficult and productivity is too low to be economical.

[0003]

On the other hand, it is known that production of gene recombinant proteins using insects or insect cells allows enzymes or useful proteins with physiological activity to be produced at high volume and relatively low cost, and that post-translational modification of the proteins is similar to that in mammals.

Specifically, high-volume production of recombinant proteins at relatively low cost is possible by a method of infecting insects or insect cells with *Baculovirus* incorporating exogenous protein genes, and physiologically active proteins marketed as pharmaceuticals are known (Japanese Unexamined Patent Publication SHO No. 61-9288, Japanese Unexamined Patent Publication SHO No. 61-9297).

[0004]

As regards production of cytokines, which are physiologically active substances with immunomodulatory action

that have attracted attention for medical uses, methods of inoculating silkworm with BmNPV incorporating the feline interferon-ω gene and the canine interferon-γ gene have been disclosed in Japanese Unexamined Patent Publication HEI No. 3-139276 and Japanese Unexamined Patent Publication HEI No. 9-234085, respectively. As an example of production of a protein other than interferon in insects, there is known a method of producing human collagen with insect cells infected with *Baculovirus* (Japanese Unexamined Patent Publication HEI No. 8-23979).

[0005]

However, since the conventional techniques for production of recombinant proteins with insects or insect cells use viruses for transfer of the exogenous genes, they must be inactivated or contained, rendering the procedure of viral inoculation more complicated. Furthermore, since the recombinant proteins are produced in body fluids, the recombinant proteins of interest must be purified from the large volumes of contaminating proteins deriving from silkworm body fluids, thereby complicating efforts to obtain highly pure recombinant proteins.

[0006]

On the other hand, recombination of exogenous genes into insect chromosomes has been attempted in recent years, and specifically a method of using DNA from *Autographa californica* nuclear polyhedrosis virus (AcNPV) to transfer a fused gene, comprising the silkworm fibroin L chain gene with the jellyfish green fluorescent protein gene, into a silkworm chromosome by homologous recombination and expressing the fused gene has been developed (Genes Dev., 13 511-516, 1999); a silkworm having the human collagen gene transferred therein and a method for its production have been developed utilizing the aforementioned technique (Japanese Unexamined Patent Publication No. 2001-161214). Recently, a method of stably transferring an exogenous gene into silkworm chromosomes using the *Lepidoptera-*

derived transposon piggyBac and expressing the protein encoded by the exogenous gene has been researched using jellyfish green fluorescent protein as a model, and it has been confirmed that the gene is stably passed to offspring by breeding (Nature Biotechnology 18, 81-84, 2000).

[0007]

However, since the green fluorescent protein obtained using AcNPV is fused with the fibroin L chain polypeptide, it has been difficult to recover the expressed recombinant green fluorescent protein alone. In addition, when the fibroin L chain polypeptide and a cytokine are produced as a fused protein by this method, reduction in physiological activity has been a problem. In cases which use the piggyBac transposon, the green fluorescent protein is not produced in sufficiently large amounts, while its production throughout the entire silkworm body means that sophisticated purification techniques must be used to recover the expressed recombinant green fluorescent protein in a highly purified form, thus presenting an economical obstacle.

[0008]

Transfer of genes coding for proteins with physiological activity, such as cytokine genes, into silkworm chromosomes and expression of the target proteins has not been hitherto known. In addition, recombinant cytokines have not been recovered from silk glands which are located at sections without silkworm body fluids or from cocoon filaments which are secreted by silkworms, nor has the physiological activity of the obtained cytokines been confirmed. Furthermore, no silkworms exist which can propagate such a nature.

In other words, production of recombinant proteins by recombination techniques for exogenous genes into insect chromosomes is still unsatisfactory, and major problems remain from the standpoint of recovering the obtained recombinant proteins in forms which retain physiological activity.

[0009]

[Problems to be Solved by the Invention]

Techniques for production of recombinant proteins using insects have been avidly researched, but they have required containment of the recombinant *Baculovirus* incorporating the exogenous genes, while inoculation with recombinant viruses is also a complicated procedure. Thus, it has been desired to produce cytokines in silkworms without using viruses. However, the production of recombinant proteins in silkworm without viruses has been hampered by the major problem that extraction and purification of target proteins from body fluids containing large amounts of impurities is difficult.

Although research has been carried out on techniques for production of recombinant proteins by transfer of exogenous genes into silkworm chromosomes, the recombinant protein productivity has been low, and the target recombinant proteins have not been recoverable in active form.

[0010]

[Means for Solving the Problems]

As a result of diligent investigation, the present inventors have found that if a DNA sequence having a structure wherein the gene coding for a protein of interest is linked downstream from a silkworm silk gland-specific expression promoter is transferred into a silkworm chromosome utilizing transposon-derived DNA, the protein of interest is produced in large volume in the silk glands or cocoon filaments, in a form retaining its physiological activity, and the present invention was thereupon completed. According to the invention, the recombinant protein can be recovered from silk glands or cocoon filaments without containing significant amounts of impurities, thereby providing the advantage of facilitating purification of the protein of interest. Furthermore, since a virus such as *Baculovirus* is not used, there is no need for virus inactivation and production of the recombinant protein can therefore be carried out in more simple and safe manner.

[0011]

In other words, the present invention relates to a method for production of recombinant cytokines characterized by creating gene recombinant silkworms incorporating cytokine genes in their chromosomes, and recovering the cytokines from the silk glands or cocoon filaments.

The invention further relates to gene-recombinant silkworms having cytokine genes incorporated therein, and to gene recombinant vectors used for transfer of the cytokine genes into silkworms.

[0012]

[Preferred Mode of the Invention]

Cytokines are proteins which are produced by various types of cells and have immunomodulatory effects on hematopoietic cells and immune cells, anti-viral effects, and hematocyte-proliferating effects. The effects are exhibited by forming precise higher structures and binding with specific receptors on cells. The features of these effects have led to their clinical application for animals including humans.

[0013]

There are no particular restrictions on the cytokines for the invention, and they may be cytokines which retain their physiological activity when expressed in silkworms, and which are proteins whose physiological activity includes immunomodulatory effects, anti-viral effects or hematocyte-proliferating effects and have noted medical or therapeutic purposes. There may be mentioned, for example, human interferon-α, β and γ (J. Interferon Res. 5, 521-526, 1985; Nucleic Acids Res. 10, 2487-2501, 1982), human interleukin-12 (J. Immunol. 146, 3074-3081, 1991), human granulocyte colony stimulating factor (Nature, 319, 415-418, 1986), human erythropoietin (Nature, 313, 806-810, 1985), human thrombopoietin (Cell 77, 1117-1124, 1994), feline interferon-ω (GenBank Accession No. E04599), feline erythropoietin (GenBank Accession No. FDU00685), canine interferon-γ (GenBank Accession No. S41201) and canine interleukin-12 (Japanese Unexamined

Patent Publication HEI No. 10-36397).

[0014]

The cytokines are preferably interferons, and more preferably feline interferon-ω. The feline interferon-ω gene may be obtained, for example, by excision from a plasmid extracted from *E. coli* (pFeIFN1) (FERM-BP 1633). The excised feline interferon-ω gene may then be linked to a silkworm cloning vector (T. Horiuchi et al., Agric. Biol. Chem., 51, 1573-1580, 1987) to create a recombinant plasmid, which with silkworm polyhedrosis virus DNA may be cotransfected into an established silkworm cell line to construct rBNV100.

[0015]

The method of gene transfer into a silkworm chromosome according to the invention may be any gene transfer method whereby a gene is stably incorporated into the chromosome and the gene is expressed and stably passed to offspring by breeding, and it may be a method of microinjection into silkworm eggs or a method of using a gene gun; however, the preferred method is one wherein a vector for exogenous gene transfer into a silkworm chromosome, comprising both a helper plasmid containing a transposon gene (Nature Biotechnology 18, 81-84, 2000) as well as the gene of interest, is microinjected into silkworm eggs.

[0016]

The gene of interest is transferred into the reproductive cells of recombinant silkworms hatched and raised from silkworm eggs subjected to microinjection. The offspring from the obtained recombinant silkworms are capable of stably retaining the gene of interest in their chromosomes. The gene ~~recombinant silkworm obtained according to the invention can be~~ subcultured by the same method as for ordinary silkworms. Specifically, the eggs may be incubated under ordinary conditions and the hatched "ant silkworms" reared on artificial feed, and raised to fifth-instar silkworms under the same conditions as for ordinary silkworms.

[0017]

The gene recombinant silkworms obtained according to the invention may be pupated in the same manner as ordinary silkworms to produce cocoons. Males and females are distinguished at the pupa stage, and after maturing to moths, the males and females are mated and the eggs harvested on the following day. The eggs may be preserved in the same manner as ordinary silkworm eggs. The gene recombinant silkworms of the invention may be subcultured by repeating this breeding method, in order to increase the volume.

The vector used for exogenous gene transfer into silkworm chromosomes may be a viral vector, a vector containing a transposon-derived DNA sequence, or a plasmid vector which is not incorporated into the chromosomes. It is preferably a vector comprising a transposon-derived DNA sequence.

[0018]

As transposon-derived DNA there may be used the *Drosophila*-derived transposon mariner (Third International workshop on transgenesis of invertebrate organisms, p37-38, 1999) and Minos (Insect Mol. Biol. 9, 277-281, 2000) or the *Lepidoptera*-derived transposon piggyBac (Nature Biotechnology 18, 81-84, 2000), but transposons having DNA sequences derived from the *Lepidoptera*-derived transposon piggyBac are preferred. The structure of a DNA sequence derived from piggyBac must include a pair of inverted terminal repeats comprising the sequence TTAA, and it has a structure wherein an exogenous gene such as a cytokine gene is inserted between those DNA sequences.

[0019]

A transposase is also preferably used for transfer of the exogenous gene into the silkworm chromosome utilizing the transposon-derived DNA sequences. For example, by simultaneous transfer of DNA which can express piggyBac-derived transposase, the transposase transcribed and translated in the silkworm cells recognizes the two pairs of inverted terminal repeats and

excises the gene fragment between them and transfers it to the silkworm chromosome, thereby notably improving the frequency of gene transfer into the silkworm chromosome.

[0020]

The exogenous gene transfer vector used for transfer of the cytokine gene of the invention into the silkworm chromosome is not particularly restricted so long as it is designed for precise control of expression of the cytokine, but ordinarily it will have a structure with a silk gland-specific expression promoter linked upstream from the cytokine gene and optionally a polyA sequence linked downstream therefrom, and with a pair of transposon-derived DNA sequences on either side of the gene sequences. In addition, an optional gene-derived signal sequence may be linked between it and the promoter, and optional gene sequences may also be linked between it and the polyA sequence.

[0021]

An artificially designed and synthesized gene sequence may also be linked. If necessary, a sequence for replication in a bacterial host, an antibiotic resistance gene, a fluorescent protein gene, a LacZ gene or the like may be linked thereto. For example, a gene for green fluorescent protein GFP linked downstream from a suitable promoter may be transferred at an appropriate site between a pair of transposon-derived DNA sequences. This will facilitate screening of the gene recombinant silkworms. The vector may also contain all or a portion of an *E. coli*-derived plasmid such as pUC9, 19, etc.

[0022]

The promoter used here is not particular restricted, and may be a promoter derived from any organism so long as it operates effectively in silkworm cells, but a promoter modified so as to induce expression of a specific protein in silkworm silk glands is preferred. As examples there may be mentioned fibroin H chain promoter, fibroin L chain promoter, p25 promoter, sericin promoter and other silkworm silk gland

protein promoters.

[0023]

As gene sequences to be used in addition to the promoter there may be mentioned signal sequences, polyA sequences, and other gene expression controlling sequences. Such sequences are not particularly restricted, and may be selected as appropriate for expression of the protein of interest. For example, there may be mentioned sequences of the protein of interest, such as signal sequences or polyA sequences of cytokines such as feline interferon-ω, or signal sequences or polyA sequences of insect proteins such as the silkworm host. In addition there may be mentioned sequences generally proven useful for proteins, such as SV40 polyA or bovine growth hormone polyA. By altering the promoter or other gene sequence linked to the cytokine gene, it is possible to manipulate the site of expression or the expression level.

[0024]

The gene recombinant silkworm used for the invention is a silkworm having a cytokine gene transferred into a silkworm chromosome, and it is a silkworm which gives a positive signal when the silkworm chromosomal DNA is treated with a restriction endonuclease by an ordinary method and the cytokine gene labeled by an ordinary method is then used as a probe for Southern blotting. The gene locus on the chromosome in which the cytokine gene is to be transferred is not particularly restricted so long as it is a position which does not inhibit development, differentiation and growth of the silkworm.

[0025]

In order to obtain an efficient yield of the gene recombinant silkworm used for the invention, a marker gene may be transferred with the cytokine gene. For example, a gene transfer vector may be used which has a green fluorescent protein gene situated in tandem with the cytokine gene between a pair of transposon sequences, and it may be injected into silkworm eggs simultaneously with a plasmid carrying a gene

coding for a transposase. The larva incubated and hatched under ordinary conditions are raised to the fifth-instar to obtain adults (G0 generation). The males and females of the G0 adults are mated to obtain eggs, and the eggs are incubated and hatched. The resulting first- to second-instar larvae, preferably, are screened for silkworms exhibiting green fluorescence, to obtain the target gene recombinant silkworms conveniently and at a high rate.

[0026]

The cytokine protein can be obtained with retained activity by using an appropriate procedure of extraction from the silk glands or cocoon filaments of the gene recombinant silkworms obtained in the manner described above. The solvent used for extraction of the cytokine from the silk glands or cocoon filaments is not particularly restricted, but is preferably an aqueous solvent system for recovery of the cytokine retaining its activity. The aqueous solution used for the extraction may contain appropriate solutes to accelerate extraction of the cytokine. As examples there may be mentioned inorganic acids such as phosphoric acid, organic acids such as acetic acid, citric acid and malic acid, salt, urea, guanidine hydrochloride, salts such as calcium chloride, and polar organic solvents such as ethanol, methanol, acetonitrile, acetone, and the like. There are no particular restrictions on the pH of the extraction solution, and any desired pH may be used so long as the cytokine of interest is not inactivated at that pH.

[0027]

There are no particular restrictions on the method of isolating and purifying the extracted cytokine, and any ordinary protein purification method may be employed. For example, purification and isolation may be accomplished by a combination of chromatography using a silica gel carrier, ion-exchange carrier, gel filtration carrier, chelating carrier, pigment-loaded carrier or the like, with ultrafiltration, gel

filtration, dialysis, desalting such as salting out, and concentration, using the original activity of the useful protein of interest as an indicator. Feline interferon-ω, for example, may be recovered from potential fractions obtained by homogenizing silk glands or cocoon filaments of silkworms into which the feline interferon-ω gene has been transferred, with 20 mM phosphate buffer solution (pH 7.0). The obtained extract may then be adsorbed onto, for example, Blue Sepharose carrier and washed, and then eluted with a salt-containing buffer solution to increase the purity of the feline interferon-ω.

[0028]

The cytokine produced in this manner may be used for medical purposes or for assay, diagnostic purposes or the like, similar to cytokines produced by other conventional production methods. When used, it may be in the form of a mixture with various additives. The tissue or cocoon filament of the silkworms expressing the cytokine may be used directly or processed for use as medical or clothing fibers.

[0029]

[EXAMPLES]

The present invention will now be explained in greater detail through examples.

Reference Example

Extraction of chromosomal DNA

After homogenizing the sample in DNA extraction buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl) containing 200 μg/ml of Proteinase K, a 1/9 volume of 10% SDS was added and reaction was conducted at 50°C for 2 hours. A portion of the reaction mixture was subjected to phenol extraction and then to ethanol precipitation by an ordinary protocol, and the precipitate was dissolved in TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) to obtain chromosomal DNA.

[0030]

Antiviral activity assay

The physiological activity of feline interferon-ω was assayed in terms of antiviral activity, by the following method.

The assay was conducted by the CPE method using Vesicular Stomatitis Virus (VSV) as the virus and Feline Fc9 (J.K. Yamamoto et al.; Vet. Immunol. and Immunopathol., 11, 1-19, 1986) as the sensitive cells. Specifically, a diluted sample solution was added to Feline Fc9 cells cultured at 37°C to confluency on a 96-well microplate, in an equal amount at the top end and with serial dilution by two-fold steps toward the bottom end.

[0031]

After culturing at 37°C for 20-24 hours, VSV was added and culturing was continued at 37°C for 16-20 hours. The surviving Feline Fc9 cells adhering to the microplate were stained with a crystal violet stain solution containing 20% formalin, and the amount of crystal violet on the microplate was measured based on the absorbance at 570 nm and compared with a standard to determine the antiviral activity. The standard used was Intercat (Toray Industries, Inc.) prepared to 1000 Unit/ml with cell culturing medium. The sample was diluted 15-fold with the cell culturing medium for use in the antiviral activity assay.

[0032]

Example 1 Gene preparation

The gene used was obtained by creating primers for both ends based on the known sequence, and conducting PCR with an appropriate DNA source as template. A restriction enzyme site was attached to the ends of the primers for subsequent gene construction and manipulation.

The feline interferon-ω gene (nucleotide bases 9-593 of GenBank Accession No. S62636) was obtained by PCR using two different primers, primer 1 (SEQ ID NO: 1) and primer 2 (SEQ ID NO: 2), with *Baculovirus* rBNV100 coding for the feline interferon-ω gene as template.

[0033]

rBNV100 may be constructed, for example, by excising the FeIFN gene from a plasmid extracted from *E. coli* (pFeIFN1) and linking it to a silkworm cloning vector (T. Horiuchi et al., Agric. Biol. Chem., 51, 1573-1580, 1987) to create a recombinant plasmid, and then cotransfecting it together with silkworm polyhedrosis virus DNA into an established silkworm cell line.

[0034]

The sericin-1 gene promoter (nucleotide bases 599-1656 of GenBank Accession No. AB007831) was obtained by PCR using two different primers, primer 3 (SEQ ID NO: 3) and primer 4 (SEQ ID NO: 4), with silkworm chromosomal DNA as template. The fibroin H chain gene promoter (nucleotide bases 255-574 of GenBank Accession No. V00094) was obtained by PCR using two different primers, primer 5 (SEQ ID NO: 5) and primer 6 (SEQ ID NO: 6), with silkworm chromosomal DNA as template. Bovine growth hormone gene polyA (nucleotide bases 1011-1253 of pcDNA3.1(+)) was obtained by PCR using two different primers, primer 7 (SEQ ID NO: 7) and primer 8 (SEQ ID NO: 8), with plasmid pcDNA3.1(+) vector (Invitrogen) as template.

[0035]

The PCR was carried out according to the manufacturer's protocol, using KODplus (Toyobo Co., Ltd.). Specifically, each template was added at 10 ng for plasmid and 100 ng for chromosomal DNA, and the reagents were added with each primer at 30 pmol, the included 10 x PCR buffer at 10 μl, 1 mM $MgCl_2$, 0.2 mM dNTPs and 2 units of KODplus, to a total of 100 μl. Reaction was carried out with 30 cycles using a Perkin-Elmer DNA thermal cycler under DNA denaturing conditions of 94°C, 15 seconds, primer annealing conditions of 55°C, 30 seconds and extension conditions of 68°C, 30-60 seconds.

[0036]

The reaction solutions were electrophoresed with 1-1.5%

agarose gel, and an ordinary procedure was used for extraction and preparation of a DNA fragment of approximately 580 bp for feline interferon-ω, approximately 1 kbp for sericin-1 promoter, approximately 320 bp for fibroin H chain promoter and approximately 230 bp for bovine growth hormone polyA. The DNA fragments were phosphorylated with polynucleotide kinase (Takara Shuzo), and after cleavage with HincII, were reacted overnight with dephosphorylated pUC19 vector at 16°C, using DNA Ligation Kit Ver.2 by Takara Shuzo Co., Ltd., for ligation. The vectors were then used to transform *E. coli* by an ordinary procedure, and insertion of the PCR fragments into the obtained transformants was confirmed by PCR of the resulting colonies under the same conditions described above, upon which the PCR fragment-inserted plasmids were prepared from the colonies by an ordinary procedure. The plasmids were sequenced to confirm that the obtained fragments had the respective gene base sequences.

[0037]

Example 2 Construction of gene transfer plasmid

Plasmid pigA3GFP (Nature Biotechnology 18, 81-84, 2000) was used as the gene transfer plasmid. Specifically, pigA3GFP is a vector obtained by removing the transposase coding region from plasmid p3E1.2 disclosed in U.S. Patent No. 6,218,185, and inserting at that section the A3 promoter (nucleotide bases 1764-2595 of GenBank Accession No. U49854), with pEGFP-N1 vector (Clontech)-derived GFP and SV40-derived polyA added sequence (nucleotide bases 659-2578 of GenBank Accession No. U55762). This vector is available from the Japan National Institute of Agrobiological Sciences.

[0038]

Feline interferon-ω gene expression sites were inserted at the *Xho*I site upstream from the A3 promoter. As the expression sites of the gene to be transferred there were used sericin-1 gene promoter/feline interferon-ω/bovine growth hormone polyA added sequence (SEQ ID NO: 1), or fibroin H chain

gene promoter/feline interferon-ω/bovine growth hormone polyA added sequence (SEQ ID NO: 2). The specific method is described below.

[0039]

The restriction enzyme sites prepared in the primers beforehand were utilized to excise the gene from the plasmid prepared in Example 1. Specifically, *Eco*RI and *Sal*I were used for the sericin-1 gene promoter and fibroin H chain gene promoter, *Sal*I and *Xba*I were used for the feline interferon-ω gene and *Xba*I and *Bam*HI were used for bovine growth hormone polyA, to excise the insert fragments which were then electrophoresed on 1-1.5% agarose gel, and the fragments were extracted and purified by ordinary methods.

[0040]

After mixing 200 ng of sericin-1 gene promoter fragment, 100 ng of feline interferon-ω gene fragment and 50 ng of bovine growth hormone polyA, an equivolume of DNA Ligation Kit Ver.2 by Takara Shuzo Co., Ltd. was added and reaction was performed overnight at 16°C. A 0.5 μl portion of the reaction mixture was subjected to PCR under extension conditions for 2 minutes, with the same conditions as in Example 1, using primer 9 (SEQ ID NO: 9) and primer 10 (SEQ ID NO: 10). The reaction solutions were electrophoresed on 1% agarose gel, and the amplified approximately 1.9 kb DNA fragment (SIB fragment) was extracted and purified by an ordinary method.

[0041]

After likewise mixing 70 ng of fibroin H chain gene promoter fragment, 100 ng of feline interferon-ω gene fragment and 50 ng of bovine growth hormone polyA, an equivolume of DNA Ligation Kit Ver.2 by Takara Shuzo Co., Ltd. was added and reaction was performed overnight at 16°C. A 0.5 μl portion of the reaction mixture was subjected to PCR under extension conditions for 2 minutes, with the same conditions as in Example 1, using primer 11 (SEQ ID NO: 11) and primer 10 (SEQ

ID NO: 10). The reaction solutions were electrophoresed on 1% agarose gel, and the amplified approximately 1.15 kb DNA fragment (SIB fragment) was extracted and purified by an ordinary method.

[0042]

These fragments were digested with *Xho*I, and then linked to *Xho*I-cleaved dephosphorylated pigA3GFP by reaction overnight at 16°C using DNA Ligation Kit Ver.2 by Takara Shuzo Co., Ltd. The SIB fragment-inserted plasmid was designated as pigFIB (Fig. 1) and the FIB fragment-inserted plasmid was designated as pigFIB (Fig. 2), and purification was performed by ultracentrifugation twice by the cesium chloride method, for use in the gene transfer experiment.

[0043]

Example 3 Creation of gene recombinant silkworm (fibroin H chain gene promoter)

Plasmid pigFIB and helper plasmid pHA3PIG (Fig. 3, Nature Biotechnology 18, 81-84, 2000, available from the Japan National Institute of Agrobiological Sciences) were each prepared to 200 ng/ml concentrations in 0.5 mM phosphate buffer (pH 7.0), 5 mM KCl, and 15-20 nl thereof was microinjected into silkworm eggs within 4 hours after egg production.

[0044]

The larva hatched from the silkworm eggs were raised, and the developed adults (G0) were cross-mated within the group to obtain a second generation (G1), which were then screened for silkworms having the feline interferon-ω gene transferred into the chromosomes based on observation of the fluorescence of green fluorescent protein transferred together with the feline interferon-ω gene. The proportion of the moth group obtained with transgenic silkworms is shown in Table 1. Injection was performed twice into the silkworm eggs, and gene recombinant silkworms were obtained from one group after the second injection.

[0045]

Table 1 Yield of gene recombinant silkworms (fibroin heavy chain promoter)

Experiment group	Number of injected eggs	Number of hatched eggs	Number of adults	Number of sib-cross matings	Number of feline interferon -ω gene-positive moth groups
1	1215	292	220	100	0
2	1326	374	250	123	1

[0046]

Fig. 4 shows the results of Southern blotting of the gene recombinant silkworms obtained from the moth group. For the Southern blotting, chromosomal DNA was extracted from the G1 generation moths and treated with restriction endonucleases and the obtained sample was electrophoresed, after which the DNA-transferred membrane was subjected to detection by chemiluminescence using an AlPhos Direct Labeling and Detection System (Amersham-Pharmacia), with a feline interferon-ω specific nucleic acid probe.

Upon examining eleven G1 moths, it was confirmed that the feline interferon-ω gene had been transferred into ten of the silkworms.

[0047]

Example 4 Confirmation of feline interferon production (fibroin H chain promoter)

Since feline interferon-ω has antiviral activity, the presence of feline interferon-ω can be determined based on titer. Of generation (G2) obtained by mating silkworms (G1) of the positive moth group obtained in Example 3 with wild silkworms, the middle silk glands and posterior silk glands were extracted from fifth-instar larvae among the silkworms in which transfer of the feline interferon-ω gene had been confirmed. The glands were homogenized with 20 mM sodium

phosphate buffer (pH 7.0), and the obtained extract was assayed by an antiviral activity assay system using feline cells. As a result, antiviral activity was detected in both the middle and posterior silk glands from the silk gland extracts of the transgenic silkworms, but was not detected from the silk gland extracts of the wild silkworm controls. The results are shown in Fig. 5.

[0048]

It is believed that feline interferon-ω was expressed mainly in the posterior silk glands under the control of fibroin H promoter, and subsequently shifted to the middle and anterior silk glands in the same manner as fibroin, with a corresponding physiological activity distribution. On the other hand, absolutely no antiviral activity was detected from the silkworms without the transferred gene. This demonstrated that in the feline interferon-ω transgenic silkworms, the feline interferon-ω protein had been expressed while retaining its physiological activity.

[0049]

Example 5 Purification of feline interferon

Feline interferon was purified from a posterior silk gland extract of the fifth-instar silkworms of generation G2 obtained in Example 4. After passing 1 ml of extract through a HiTrap Blue Sepharose column (Amersham-Pharmacia), it was washed with 10 ml of 20 mM sodium phosphate buffer (pH 7.0). Elution was then performed with 10 ml of 20 mM sodium phosphate buffer (pH 8.0)-0.5 M NaCl, and subsequently with 10 ml of 20 mM sodium phosphate buffer (pH 8.0)-1 M NaCl. The wash fraction, 0.5 M elution fraction and 1 M elution fraction were divided and subjected to desalting and concentration to obtain approximately 1 ml. Table 2 shows the results for the antiviral activities and protein quantities of the extract and each purified fraction.

[0050]

Table 2 Purification of feline interferon-ω by Blue Sepharose chromatography

	Antiviral activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)
Extraction sample	523	0.37	1401
Flow-through/wash fraction	23	2.91	8
0.5 M NaCl elution fraction	1494	0.85	1758
1 M NaCl elution fraction	≥6270	0.41	≥15,293

[0051]

The purification procedure yielded antiviral activity, i.e. feline interferon-ω in the 1 M elution fraction, and the specific activity was approximately 10 times that of the extract.

[0052]

Example 6 Creation of gene recombinant silkworms (sericin-1 promoter)

Plasmid pigFIB and the helper plasmid were each prepared to 200 ng/ml concentrations in 0.5 mM phosphate buffer (pH 7.0) + 5 mM KCl, and 15-20 nl thereof was microinjected into silkworm eggs within 4 hours after egg production. The larvae which hatched from the silkworm eggs were raised, and the developed adults (G0) were cross-mated within the group to obtain a second generation (G1), which were then examined for transfer of the feline interferon-ω gene into the chromosomes based on observation of the fluorescence of green fluorescent protein. Two experiments were conducted with microinjection of a gene recombinant vector containing the feline interferon-ω gene linked to sericin promoter into 1218 and 1375 eggs,

respectively, and this yielded 12 positive moth groups (Table 3).

[0053]

Table 3 Yield of gene recombinant silkworms (sericin promoter)

Experiment group	Number of injected eggs	Number of hatched eggs	Number of adults	Number of sib-cross matings	Number of feline interferon -ω gene-positive moth groups
1	1218	500	320	158	12
2	1375	540	500	225	12

[0054]

Of the obtained positive moth groups, one silkworm (G1) confirmed to have the transferred gene was selected from each of 3 moth groups of the first experiment and 2 moth groups of the second experiment, and genomic DNA was extracted from the silk glands. After treatment with *Eco*RI or *Bgl*II, the feline interferon-ω gene was used as a probe for Southern blotting analysis, giving the results shown in Fig. 6. Transfer of the feline interferon-ω gene was confirmed in the genomes of all of the silkworms. Due to differences in the detected positions, it was also found that the gene transfer sites into the genomes differed depending on the moth group.

[0055]

Next, mRNA expression of the feline interferon-ω gene was examined. Seven G1 generation silkworms in which transfer of the feline interferon-ω gene had been confirmed by Southern blotting were arbitrarily selected, the mRNA was extracted, and expression of feline interferon-ω gene mRNA was examined by RT-PCR. The mRNA extraction and purification was carried out using ISOGEN (Nippon Gene) and Oligotex dT-30 (Roche Diagnostics) and cDNA synthesis was carried out using Ready-To-Go T-Primed First-Strand Kit (Amersham-Pharmacia), according to the manufacturer's protocol. The PCR was carried out under the

conditions for obtaining the feline interferon-ω in Example 1, by which expression of feline interferon-ω gene mRNA was confirmed by all individuals (Fig. 7).

[0056]

Example 7 Confirmation of feline interferon-ω production in middle silk glands and cocoon filaments

The middle silk glands were extracted from three of the gene recombinant silkworms obtained in Example 6 and from one wild silkworm, and then were homogenized with 20 mM sodium phosphate buffer (pH 7.0) and centrifuged to prepare extracts. One cocoon each was also extracted from the gene recombinant silkworms and wild silkworm. Upon assaying the antiviral activity of each of the extracts, antiviral activity was detected in the middle silk glands of all of the gene recombinant silkworms, but not in the silk glands of the wild silkworm. Antiviral activity was also detected in the cocoons of the gene recombinant silkworms (Fig. 8).

[0057]

This indicated that the feline interferon-ω had been expressed while retaining physiological activity in the gene recombinant silkworms, and that its activity still is exhibited when spun into filaments.

[0058]

[Effect of the Invention]

It has become possible to obtain a physiologically active cytokine at high yield from the silk glands or cocoon filaments of gene recombinant silkworms obtained by gene transfer into silkworm chromosomes using a plasmid vector comprising the cytokine gene linked with a promoter which functions in silkworm silk glands. The obtained cytokine extract contains few contaminating proteins and its purification is simpler than by prior art methods.

[0059]

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[BRIEF DESCRIPTION OF THE DRAWINGS]

Fig. 1 is a restriction enzyme map of the gene transfer vector pigSIB.

Fig. 2 is a restriction enzyme map of the gene transfer vector pigFIB.

Fig. 3 is a restriction enzyme map of the transposase-carrying plasmid pHA3PIG.

Fig. 4 is a photograph showing the results of *Eco*RV and *Xmn*I treatment of the genomic DNA of eleven silkworms (G1)

obtained from the positive moth group of Table 1, followed by Southern blotting using the feline interferon-ω gene as a probe.

Fig. 5 is a photograph showing antiviral activity of silk extracts from recombinant silkworms in which the feline interferon-ω gene linked to the fibroin H chain promoter had been transferred. Samples in the stained lanes are those exhibiting activity.

Fig. 6 is a photograph showing the results of *Eco*RI and *Bgl*II treatment of silkworm silk gland genomic DNA from the positive moth groups of Table 3 (3 moth groups from experiment 1 and two moth groups from experiment 2), followed by Southern blotting using the feline interferon-ω gene as a probe.

Fig. 7 is a photograph showing RT-PCR detection of feline interferon-ω mRNA expression in gene recombinant silkworm middle silk glands.

Fig. 8 is a photograph showing antiviral activity of middle silk gland extracts and cocoon filament extracts from recombinant silkworms in which the feline interferon-ω gene linked to the fibroin H chain promoter had been transferred. Samples in the stained lanes are those exhibiting activity.

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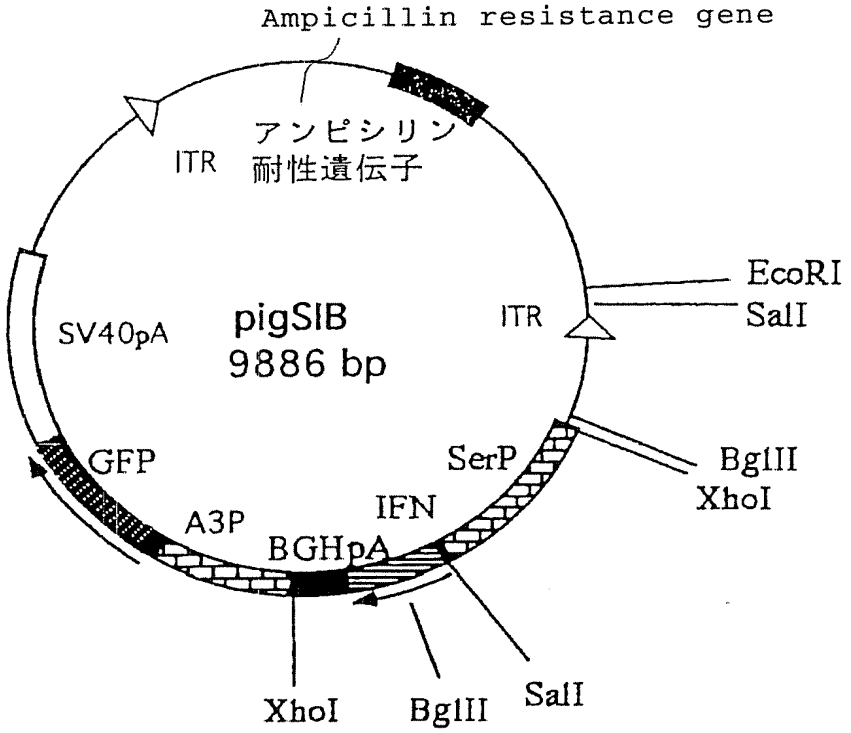
【Name of Document】　Drawings
【図１】
【Fig. 1】

図1
Fig. 1



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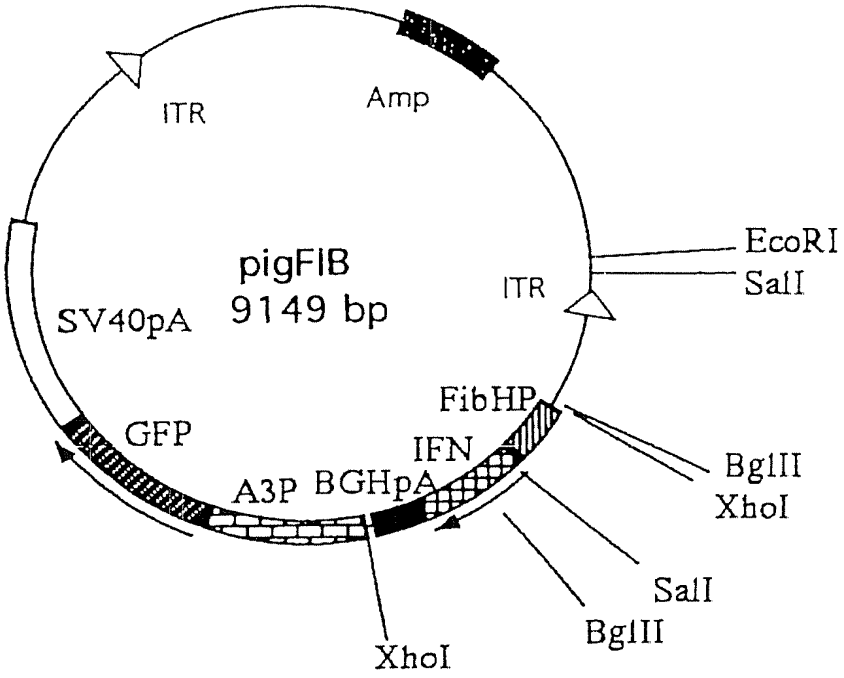
ＢＧＨｐＡ：ウシ成長ホルモンポリＡ
Ａ３Ｐ　：Ａ３プロモーター
ＧＦＰ　：緑色蛍光タンパク質
ＳＶ40ｐＡ：ＳＶ40ポリＡ
ＩＴＲ　：末端逆位配列

SerP : Sericin-1 gene promoter
IFN : Feline interferon-ω gene
BGHpA: Bovine growth hormone polyA
A3P : A3 promoter
GFP : Green fluorescent protein
SV40pA: SV40 polyA
ITR : Inverted terminal repeat

【図２】
【Fig. 2】

図2
Fig. 2



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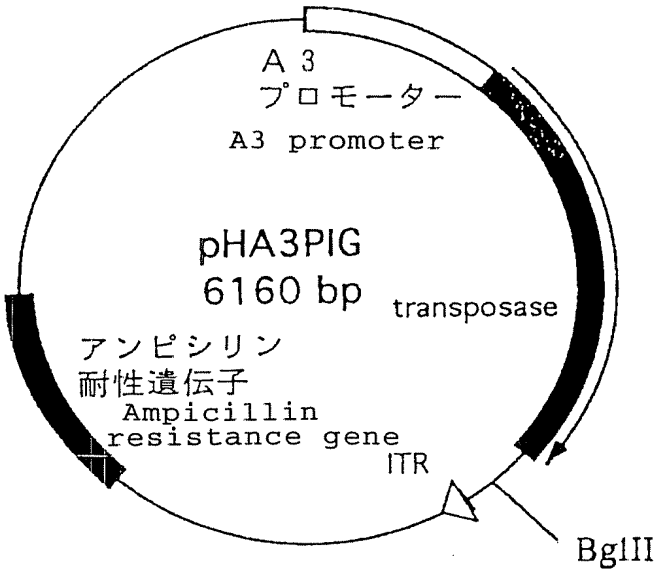
ＢＧＨｐＡ：ウシ成長ホルモンポリＡ
Ａ３Ｐ　　：Ａ３プロモーター
ＧＦＰ　　：緑色蛍光タンパク質
ＳＶ40ｐＡ：ＳＶ40ポリＡ
ＩＴＲ　　：末端逆位配列

FibHP : Fibroin H chain gene promoter
IFN　 : Feline interferon-ω gene
BGHpA : Bovine growth hormone polyA
A3P　 : A3 promoter
GFP　 : Green fluorescent protein
SV40pA: SV40 polyA
ITR　 : Inverted terminal repeat

【図３】
【Fig. 3】

図3
Fig. 3



ＩＴＲ：末端逆位配列
ITR　: Inverted terminal repeat

【図４】
【Fig. 4】　Confirmation of transgenic silkworms by Southern blotting (fibroin heavy chain promoter)

図4　遺伝子導入カイコのサザンブロッティング
Fig. 4　による確認（フィブロイン重鎖プロモーター）

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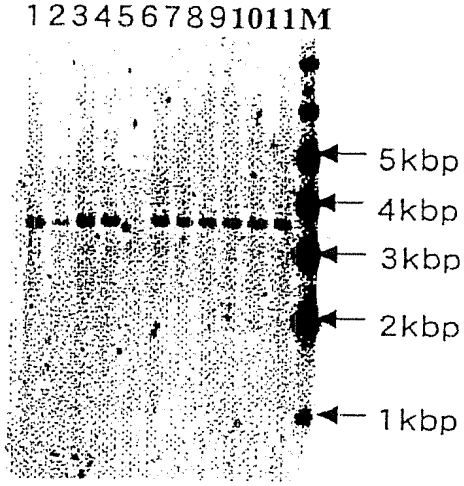


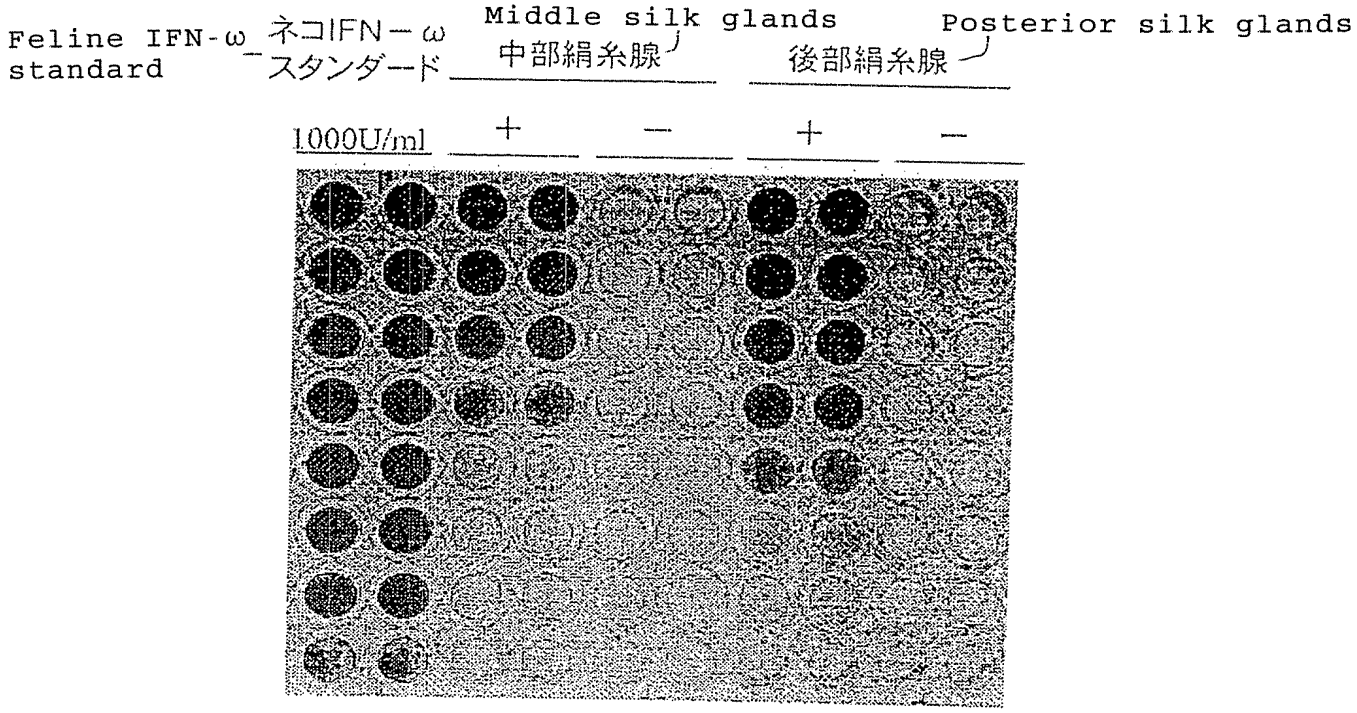
M:分子量マーカー

M: Molecular weight marker

【図５】
【Fig. 5】

Antiviral activity in silk glands of feline interferon-ω transgenic silkworms (fibroin heavy chain promoter)

図5　ネコインターフェロンーω遺伝子導入カイコ
Fig. 5　の絹糸腺における抗ウイルス活性
（フィブロイン重鎖プロモーター）



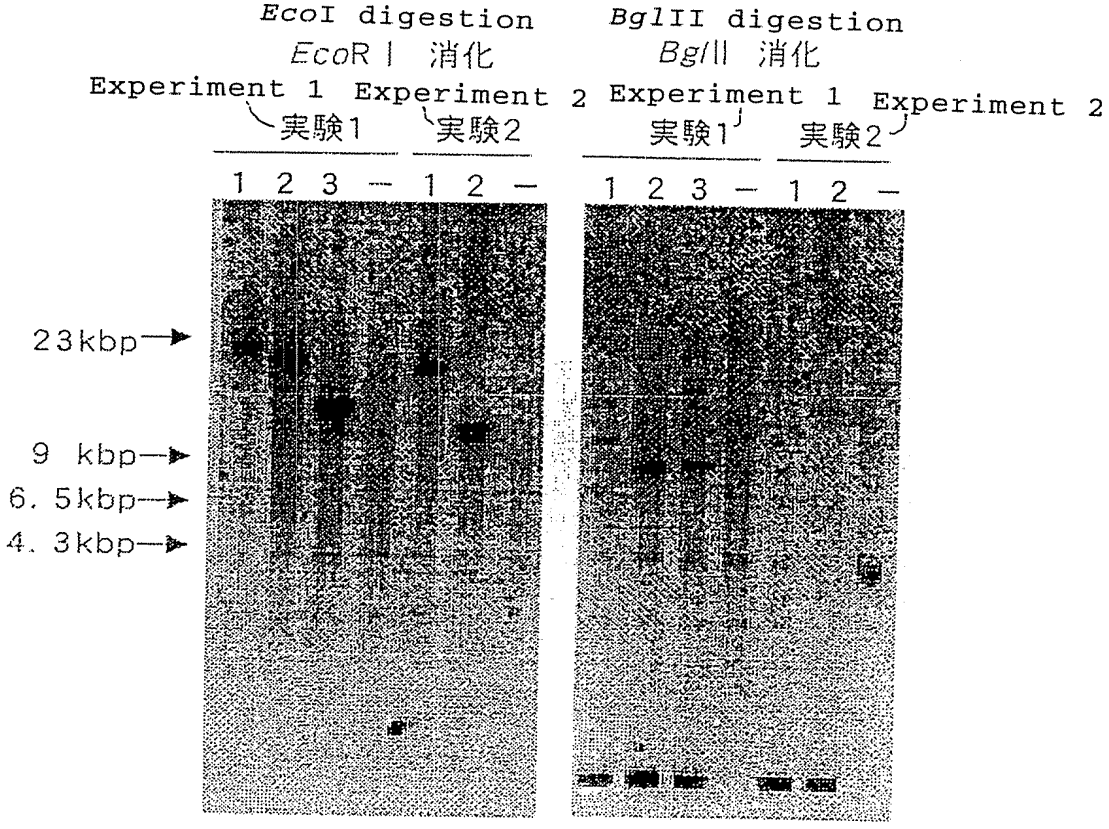
＋:遺伝子導入カイコ
ー:コントロールカイコ

+: Transgenic silkworms
-: Control silkworms

【図６】
【Fig. 6】

Confirmation of transgenic silkworms by Southern blotting (sericin promoter)

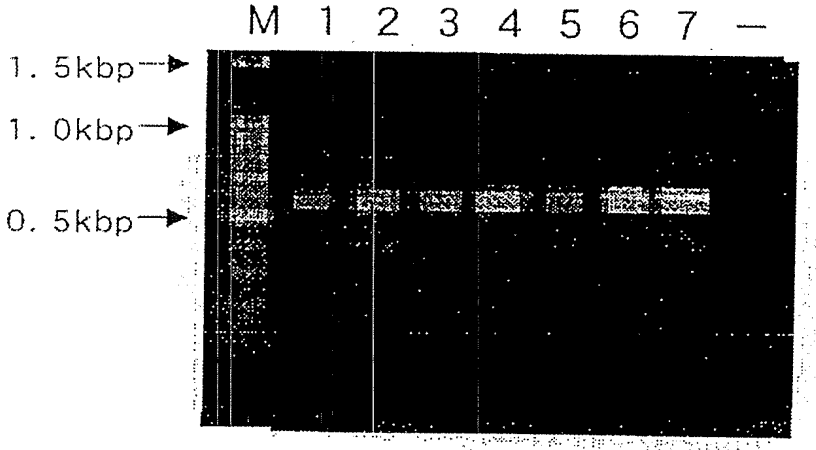
図6　遺伝子導入カイコのサザンブロッティング
Fig. 6　による確認（セリシンプロモーター）



【図７】
【Fig. 7】

Fig. 7　Expression of mRNA in feline interferon-ω transgenic silkworms (RT-PCR)

図7　ネコインターフェロンーω遺伝子導入カイコにおけるmRNAの発現（RT－PCR）



M:分子量マーカー

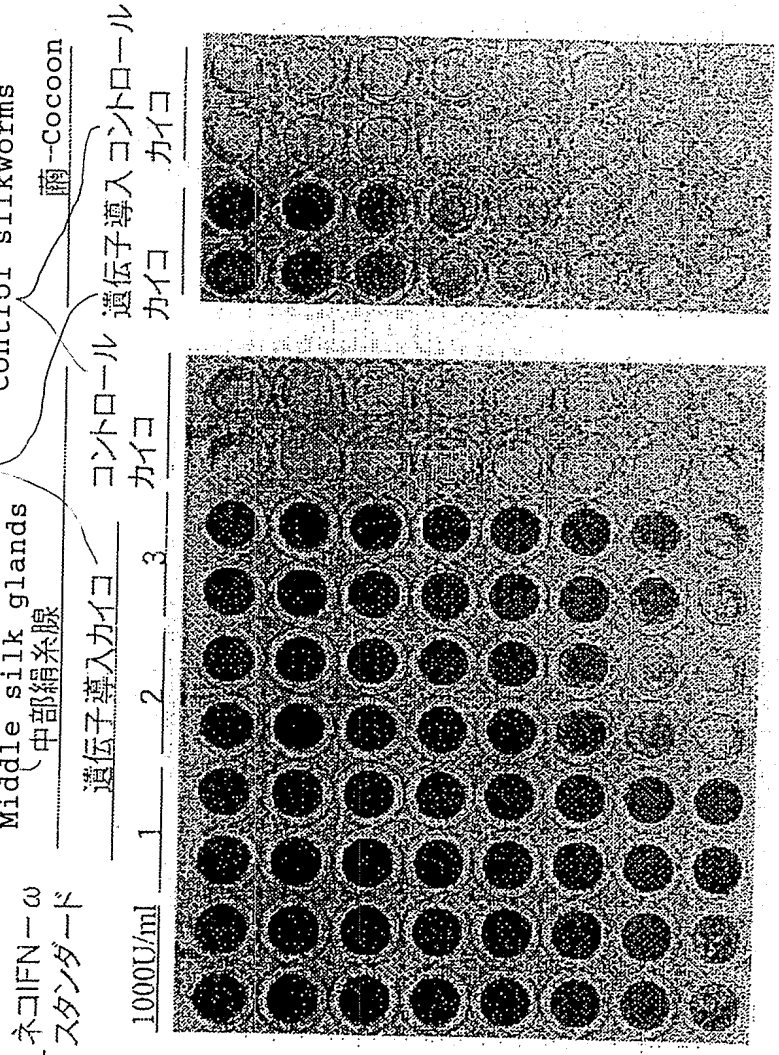
M: Molecular weight marker

【図８】
【Fig. 8】

Fig. 8 図８

Antiviral activity in silk glands and cocoons of feline interferon-ω transgenic silkworms (sericin promoter)

ネコインターフェロン－ω遺伝子導入カイコの中部絹糸腺および繭における抗ウイルス活性（セリシンプロモーター）



[NAME OF DOCUMENT] ABSTRACT

[SUMMARY]

[OBJECT]

To allow convenient manufacture of high purity feline interferon by production of the recombinant cytokine in active form in the silk glands or cocoon filaments of silkworms.

[SOLUTION MEANS]

A gene recombinant vector, comprising a gene sequence which includes the cytokine gene linked to a promoter which is functional in silk glands and a transposon-derived sequence and other sequences necessary for gene transfer into silkworms, is used for transfer of the cytokine gene into a silkworm chromosome to obtain a gene recombinant silkworm. The cytokine is extracted and purified from the silk glands or cocoons of the silkworm.

[SELECTED DRAWING] None